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Summarizing discussion

Multiple Sclerosis (MS) is a well known chronic inflammatory disease of the central nervous system (CNS) that mainly affects young adults. Much research is performed to understand and develop treatment of MS which has led to the introduction of several medications for MS. However, these treatments do not cure MS and are troubled by side effects. The failure of current forms of therapy to stop progression of the disease and their lack of specificity therefore provides strong impetus to continue the search for more effective and less toxic therapies. During the initial phase of relapsing-remitting MS, the infiltration and migration of leukocytes into the CNS is a major pathological process. When MS progresses, there are also other neuropathological features including astrogliosis and failure of remyelination, resulting in neurodegeneration and long-lasting neurological deficits. Therefore, it is essential to characterize molecular and cellular events that underlie the different neuropathological processes occurring during MS. This may ultimately lead to the identification of novel therapeutic targets to combat the various neuropathological processes contributing to MS.

The studies described in this thesis were aimed to investigate the contribution of tissue Transglutaminase (TG2) to various neuropathological processes underlying MS. TG2 is a multifunctional enzyme that is ubiquitously expressed in the human body. It is a calcium dependent enzyme which can catalyze post-translational modification of proteins by cross-linking via $\gamma(\epsilon\text{-glutamyl})$ lysine bonds. Moreover, TG2 has been shown to be a GTP-binding protein and has GTPase activity. TG2 is localized in the cytoplasm, on the cell surface and in the extracellular matrix (ECM), where it plays an important role in cell-matrix interactions. Of the transglutaminase family, TG1, TG2 and TG3 have been shown to be expressed in the healthy human CNS. TG2 has been implicated in various (patho)physiological processes, including cell adhesion and migration, extracellular matrix formation, cell apoptosis and survival, and cell differentiation. As described in chapter 1, MS is characterized by infiltration of immune cells into the CNS, production of inflammatory mediators, demyelination, astrogliosis, failure of remyelination, and subsequent axonal damage. Since TG2 is expressed in the human CNS, and has been shown to be involved in a number of cellular processes, including cell adhesion and migration, apoptosis, cell differentiation and inflammation, which contribute to MS pathology, we hypothesized that TG2 plays a crucial role in neuropathological processes underlying MS including; 1) monocyte infiltration and migration into the CNS (chapter 3), 2) glial scar formation (chapters 4 and 5), and 3) differentiation of oligodendrocyte precursor cells (OPCs) and subsequent (re)myelination (chapter 6). Although the results are discussed separately in each chapter, I will summarize them here, and put the most important observations in perspective to draw conclusions about their importance for future development of improved therapy.

TG2 expression in human MS lesions and in the CNS of cr-EAE animals

To be able to quantitatively measure TG2 protein levels in human and rodent tissue and cells, a TG2 specific sandwich enzyme-linked immunosorbent assay (ELISA) was developed as described in chapter 2. This study was the first to describe a quantitative TG2 protein measurement across species and across matrices containing TG2 protein. This assay is of considerable interest for the research described within this thesis, but also for other research groups working on TG2.

We started our studies on the role of TG2 in MS by determining alterations in TG2 immunoreactivity in human post-mortem material of MS patients and in fresh frozen material from the CNS of animals suffering from experimental MS. As described in chapter 3, TG2 immunoreactivity appeared in infiltrating monocytes/macrophages in active and chronic active MS lesions, and in the spinal cord of rats suffering from chronic relapsing experimental autoimmune encephalomyelitis (cr-EAE), a widely used animal model for MS. Expression levels of other transglutaminase family members that have been shown to be present in the human brain, i.e. TG1 and TG3, were unaltered in human active MS lesions and in the spinal cord of cr-EAE animals compared to healthy controls (data not shown).

Thus, as described in chapter 3, TG2 is present in infiltrating monocytes around blood vessels in active MS lesions and in the spinal cord of cr-EAE rats. Furthermore, in chapter 4, it was shown that TG2 also colocalizes with glial fibrillary acidic protein (GFAP), a marker for reactive astrocytes, in active and chronic active MS lesions. Interestingly, in contrast to MS lesions, in the spinal cord of cr-EAE rats, TG2 is mainly expressed in infiltrating monocytes, but only observed occasionally in GFAP positive astrocytes (chapter 3). This might be due to species differences or staining problems since we show in chapter 5 that TG2 expression and activity is increased in primary rat astrocytes upon cytokine treatment *in vitro*.

TG2 determines the clinical outcome of experimental MS

To study if TG2 plays a clinically relevant role, we reduced TG2 activity during cr-EAE using the highly specific small molecule inhibitor KCC009. Inhibition of TG2 activity resulted in an immediate and dramatic reduction of clinical scores, even when treatment with KCC009 was started only during ongoing disease. Also use of cystamine, a less specific TG2 inhibitor, from first signs of cr-EAE onwards, induced less pronounced, but significant clinical improvement. These observations suggest that TG2 activity is of clinical importance, and is a potential novel therapeutic target for the treatment of MS.

TG2 facilitates monocyte infiltration into the CNS

Previous studies showed that TG2 expression is present in monocytes and TG2 activity is involved in monocyte differentiation into macrophages. Moreover, upon binding of monocytes to endothelial cells, TG2 gene expression was increased suggesting that TG2 also is involved in subsequent monocyte migration across endothelial cell layers. Furthermore, it is thought that TG2 plays a major role in cell adhesion and migration processes. In fact, TG2 expression on the surface of monocytes serves as an integrin-associated co-receptor for adhesion to and migration on the ECM protein fibronectin. Besides the direct evidence that TG2 is involved in adhesion and migration of cells, also observations indirectly related to cell migration have been presented. For example, TG2 can regulate the activity of matrix metalloproteases (MMPs), which are proteins that are involved in cell migration. Furthermore, TG2 was shown to be involved in cytoskeletal remodeling, which is essential for cell adhesion and migration. Thus, the presence of TG2 in infiltrating monocytes/macrophages, as shown in chapter 3, suggests that TG2 is involved in monocyte/macrophage adhesion onto endothelial cells, and subsequent infiltration and migration into the CNS.

The data described in chapter 3 of this thesis are the first to illustrate that TG2 is indeed involved in monocyte adhesion to and migration across endothelial cells in the spinal cord of cr-EAE rats *in vivo* and were confirmed by using several dedicated *in vitro* cell models. To determine the role of TG2 in monocyte infiltration, we inhibited TG2 activity *in vivo* and *in vitro* using the previously mentioned TG2 activity inhibitor KCC009. Upon inhibition of TG2 activity from different stages of cr-EAE onwards, monocyte adhesion onto brain endothelium appeared to be increased whereas monocyte migration into the CNS was significantly reduced indicating that TG2 is particularly important for monocyte migration across endothelial cells. Interestingly, KCC009 treatment of cr-EAE animals reduced TG activity selectively in spinal cord and not in cervical lymph nodes or spleen although recruitment of activated monocytes to these organs has been observed in MS and EAE. These data suggest that treatment with KCC009 locally inhibits TG2 activity at a site where it is most pronounced during cr-EAE, i.e. at the interface between the blood and the CNS. Although MS and EAE are considered to be T-cell mediated diseases, no TG2 activity was detected in T-cells isolated from cr-EAE animals, and no effect on T-cell migration into the spinal cord of cr-EAE animals was observed after KCC009 treatment, suggesting that TG2 is not involved in T-cell infiltration.

The data presented in chapter 3 demonstrate that TG2 activity, exhibited by activated monocytes but not by T-cells, is a key element in regulating monocyte adhesion and migration into the CNS during cr-EAE, thereby determining the clinical outcome. This is in line with previous studies where it was shown that activated monocytes and macrophages play a crucial role in the neuropathology and clinical outcome of EAE.

Neuropathological studies of the spinal cord of KCC009 treated cr-EAE animals showed that activated monocytes accumulated within the lumen of blood vessels and in the perivascular space with far less cells migrating into the CNS parenchyma compared to vehicle-treated cr-EAE animals. These findings were confirmed by subsequent *in vitro* studies using monocytes adhering onto and migrating over a brain endothelial cell layer.

As shown in chapter 3, TG2 regulates monocyte adhesion and migration via activation of RhoA, thereby inducing cytoskeletal rearrangements. RhoA belongs to the Rho family of small G-proteins and is crucial in regulation of cell morphology, cell differentiation and cytoskeletal rearrangement. RhoA is a substrate for TG2. In fact, activation of TG2 leads to transamidation of RhoA and after transamidation, RhoA is activated and binds/activates RhoA-associated kinase-2 (ROCK-2), a downstream target of GTP-bound Rho. This RhoA-ROCK-2 complex is transported to the cell surface to increase formation of stress fibers and focal adhesion complexes.

In addition to the effects of KCC009 on monocyte adhesion and migration, we observed also that inhibition of TG2 activity by KCC009 resulted in reduced demyelination in the spinal cord of cr-EAE animals. This is certainly of clinical interest, and may be due to the fact that iNOS and TNF α expression was reduced in the spinal cord. In MS, macrophages are the effector cells in active demyelination, primarily by production of iNOS and TNF α . Again, this effect of TG2 inhibition seemed to be restricted to the spinal cord, as the expression levels of iNOS and TNF α in the spleen of cr-EAE animals were not affected. By treatment with KCC009, however, the expression levels of other inflammatory mediators, including pro- and anti-inflammatory cytokines and chemokines, remained unaltered in both

spinal cord and spleen of KCC009 treated animals versus vehicle treated EAE animals, suggesting that infiltrating macrophages are not the primary source of those mediators. Of additional interest is that the activation status of local microglial cells, as measured by CD68 mRNA expression, a marker for activated microglia and macrophages, was not different between KCC009 treated or vehicle treated cr-EAE animals, perhaps accounting for at least part of the production of inflammatory mediators.

Astrocyte-derived TG2 interacts with fibronectin

In the active phase of MS, astrocytes become activated, migrate, and contribute to local tissue remodeling that ultimately results in the so-called astroglial scar. This reactivity of astrocytes is accompanied by changes in expression of e.g. adhesion molecules, cytokines, proteoglycans and proteases thereby creating an environment which may impede tissue repair. Therefore, astroglial scar formation is considered as a major mechanical impediment to remyelination and axonal regeneration in MS. Likely, the formation of the astroglial scar is facilitated by deposition of extracellular matrix proteins, including fibronectin. Indeed, an increased expression of various ECM proteins at the lesion sites in MS has been described.

An example of TG2's role in tissue remodeling has been shown in glioblastomas. Reduction of TG2 activity by KCC009 disrupts the assembly of fibronectin fibrils in the extracellular matrix in glioblastomas. This results in increased apoptosis of tumor cells after chemotherapy. Furthermore, TG2 has been implicated in adhesion and migration of fibroblasts and monocytic cells onto extracellular matrix proteins, in particular fibronectin.

In this thesis, in chapters 4 and 5, we showed that TG2 can mediate the interaction between astrocytes and fibronectin. At first we observed that TG2 immunoreactivity appears in reactive astrocytes in and at the rim of human MS lesions (chapter 4). This TG2 at least partly colocalized with intracellular or extracellular fibronectin. We subsequently studied the role of TG2 in adhesion and migration of astrocytes onto fibronectin. Indeed, downregulation of TG2 by siRNA or inhibition of its activity by KCC009 reduced astrocyte adhesion and migration, suggesting a role for TG2 in regulation of astrocyte motility (chapter 4). An additional *in vitro* study further extended these observations. Thus, as shown in chapter 5, TG2 expression, inside cells and present at the surface of astrocytes, is regulated by inflammatory cytokines. Moreover, these *in vitro* experiments showed that cell surface TG2 is clearly involved in the interaction of astrocytes with the extracellular matrix protein fibronectin and thereby modulates astrocyte adhesion to fibronectin.

Similar to monocytes/macrophages, we show that TG2 is an essential factor in regulating RhoA GTPase activity in astrocytes to stimulate focal adhesion formation necessary for interaction of astrocytes with the ECM and astrocyte migration. These focal adhesions link the extracellular matrix to the cytoskeleton via integrin receptors and adapter proteins such as vinculin. Focal adhesions can transmit signals into the cell via activation of focal adhesion kinase (FAK). In astrocytes, TG2 is involved in focal adhesion formation and this is mediated by phosphorylation of FAK and expression of vinculin. Therefore, we conclude that TG2 activity at the surface of astrocytes is involved in binding to fibronectin. This might result in fibronectin remodeling that leads to stabilization of the ECM to facilitate glial scar formation. Since it has been shown in EAE mice that migration of OPCs remained restricted to the margins of the lesions and in chronic MS lesions a reduced number of OPCs were present compared to non-lesioned areas, I suggest that astrocyte-ECM interactions, mediated at least partially by TG2, could impair oligodendrocyte migration and thereby remyelination.

TG2 is involved in differentiation of oligodendrocyte precursor cells and remyelination

As an alternative to the astroglial scar theory, it has been proposed that remyelination failure in MS is due to a lack of factors stimulating differentiation of OPCs into myelinating oligodendrocytes. Simultaneous to demyelination, remyelination occurs in MS lesions. This remyelination, however, is often not fully successful. Despite partial, spontaneous myelin repair in MS giving rise to so-called shadow plaques, most OPCs remain in an undifferentiated state resulting in a failure to generate mature myelinating oligodendrocytes.

TG2 is involved in different cellular functions including cell differentiation and apoptosis. With regard to cellular differentiation, it has been shown that TG2 can enhance neuronal differentiation via activation of RhoA GTPase activity. As shown in chapter 6, TG2 contributes to differentiation of OPCs into myelin-producing oligodendrocytes. TG2 expression is present in OPCs and reduced when these cells differentiated into more mature and myelinating oligodendrocytes. Interestingly, TG2 is also present in PDGFR α positive OPCs in the developing human cerebellum at gestational week 28 when OPCs start to differentiate. Inhibition of TG2 activity *in vitro* reduced the differentiation of OPCs into mature oligodendrocytes. Furthermore, *in vivo* experiments using TG2 knockout and wild-type mice in the cuprizone model for de- and remyelination showed that TG2 is involved in remyelination since myelin formation

lagged behind in TG2 knockout mice. This impaired remyelination in TG2 knockout mice had negative consequences for the recovery of motor-performance of these animals. Reduced remyelination in TG2 knockout mice correlated with impaired motor-performance, which is in line with previous studies where it was shown that demyelination in the corpus callosum correlated with motor function of rodents.

Investigation of the underlying mechanism(s) points to a role for RhoA GTPase activity in TG2-mediated OPC differentiation. It is known that Rho-GTPases are expressed by OLGs and their activity is essential for promoting process extension during OPC differentiation. Since TG2 is clearly present in OPCs, as shown in chapter 6, and TG2 can transamidate RhoA GTPase thereby increasing its activity, we expected that inhibition of TG2 activity would attenuate RhoA activity. Indeed, RhoA activity was reduced in OPCs upon KCC009 treatment. Our data suggest that, in OPCs, TG2 plays an intracellular role. During OPC differentiation into more mature oligodendrocytes, intracellular calcium levels increase, which can result in activation of TG2. Upon transamidation of RhoA by TG2, activated RhoA GTPase enhances the activity of the mitogen-activated protein kinase (MAPK) pathway, which in its turn is involved in expression of myelin specific genes that regulate OLG elongation and branching. In this manner, TG2 may play an important role in OPC differentiation and probably acts by upregulating RhoA activity which subsequently activates intracellular pathways, e.g. the MAPK pathway, thereby promoting differentiation of OPCs into myelin forming OLGs.

General conclusions

The data described in this thesis support our original hypothesis and provide the first evidence that TG2 has a multifaceted role in the neuropathology of MS.

In active human MS lesions and in the spinal cord of cr-EAE animals, TG2 expression is induced and expressed by monocytes/macrophages. *In vivo* analysis and subsequent *in vitro* studies showed that TG2 is essential for monocyte infiltration into the CNS. This will result in the presence of macrophages in the CNS which express pro-inflammatory factors such as iNOS and TNF α , leading to demyelination. Thus, due to its role in monocyte/macrophage infiltration, TG2 is indirectly involved in demyelination.

In active and chronic active MS lesions, TG2 is expressed by astrocytes. Subsequent *in vitro* studies showed that TG2 is present on the surface of astrocytes and increased after treatment with pro-inflammatory cytokines. The data described in this thesis suggests that TG2 can contribute to astrogliosis by mediating the adherence between (reactive) astrocytes and fibronectin, an ECM protein upregulated in MS lesions. Based on previous findings, e.g. that tumor cell derived TG2 is involved in remodeling of fibronectin in the ECM, I suggest that in MS lesions TG2 is active in ECM remodeling and glial scar formation. These actions of TG2 likely impair remyelination.

On the other hand, TG2 is present in OPCs and stimulates differentiation of these precursor cells into mature, myelinating oligodendrocytes, thereby positively contributing to (re)myelination.

The exact role of TG2 in MS pathology is thus dependent on the cell type which is expressing TG2, the activation state of this cell type and the intracellular, cell surface or extracellular localization of TG2.